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INACTIVATION OF THE DNA-REPAIR GENE *MGMT* AND THE CLINICAL RESPONSE OF GLIOMAS TO ALKYLATING AGENTS

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AND JAMES G. HERMAN, M.D.

ABSTRACT

Background The DNA-repair enzyme O⁶-methylguanine-DNA methyltransferase (*MGMT*) inhibits the killing of tumor cells by alkylating agents. *MGMT* activity is controlled by a promoter; methylation of the promoter silences the gene in cancer, and the cells no longer produce *MGMT*. We examined gliomas to determine whether methylation of the *MGMT* promoter is related to the responsiveness of the tumor to alkylating agents.

Methods We analyzed the *MGMT* promoter in tumor DNA by a methylation-specific polymerase-chain-reaction assay. The gliomas were obtained from patients who had been treated with carmustine (1,3-bis(2-chloroethyl)-1-nitrosourea, or BCNU). The molecular data were correlated with the clinical outcome.

Results The *MGMT* promoter was methylated in gliomas from 19 of 47 patients (40 percent). This finding was associated with regression of the tumor and prolonged overall and disease-free survival. It was an independent and stronger prognostic factor than age, stage, tumor grade, or performance status.

Conclusions Methylation of the *MGMT* promoter in gliomas is a useful predictor of the responsiveness of the tumors to alkylating agents. (N Engl J Med 2000; 343:1350-4.)

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ALKYLATING agents are highly reactive molecules that cause cell death by binding to DNA.^{1,2} The most frequent site of alkylation in DNA is the O⁶ position of guanine. Alkylation here forms cross-links between adjacent strands of DNA,¹ which explains how the nitrosoureas, tetrazines, and procarbazine kill cells. The cross-linking of double-stranded DNA by alkylating agents is inhibited by the cellular DNA-repair protein O⁶-methylguanine-DNA methyltransferase

(*MGMT*), also known as O⁶-alkylguanine-DNA alkyltransferase. The *MGMT* protein rapidly reverses alkylation at the O⁶ position of guanine,^{3,4} thereby averting the formation of lethal cross-links. Through this mechanism, *MGMT* causes resistance to alkylating drugs.^{3,4}

The level of *MGMT* varies widely according to the type of tumor, and even varies among tumors of the same type. For example, approximately 30 percent of gliomas lack *MGMT*.^{5,6} This deficiency of the enzyme may increase the sensitivity of brain tumors to alkylating agents.⁷⁻⁹ Because the *MGMT* gene is not commonly mutated or deleted, a lack of *MGMT* may be caused by changes that do not alter the genetic information of the cell. Methylation of DNA is the main type of such epigenetic modifications in humans,¹⁰ and it plays an important part in tumorigenesis. In particular, methylation of normally unmethylated sites, known as CpG (cytidine phosphate guanosine) islands, in the promoter regions of tumor-suppressor and DNA-repair genes is correlated with loss of expression of these genes in cancer cell lines and primary tumors.¹⁰ Methylation of the CpG island in the *MGMT* gene prevents transcription of the gene, and in cell lines that cannot repair alkylation of O⁶-methylguanine, the promoter of *MGMT* is methylated.¹¹⁻¹⁴ Furthermore, in vitro treatment with demethylating drugs restores the expression of the *MGMT* gene in such cells (Fig. 1).^{11,15}

We performed a study to determine whether methylation of the promoter region of the *MGMT* gene could be used to identify gliomas that were responsive to alkylating drugs.

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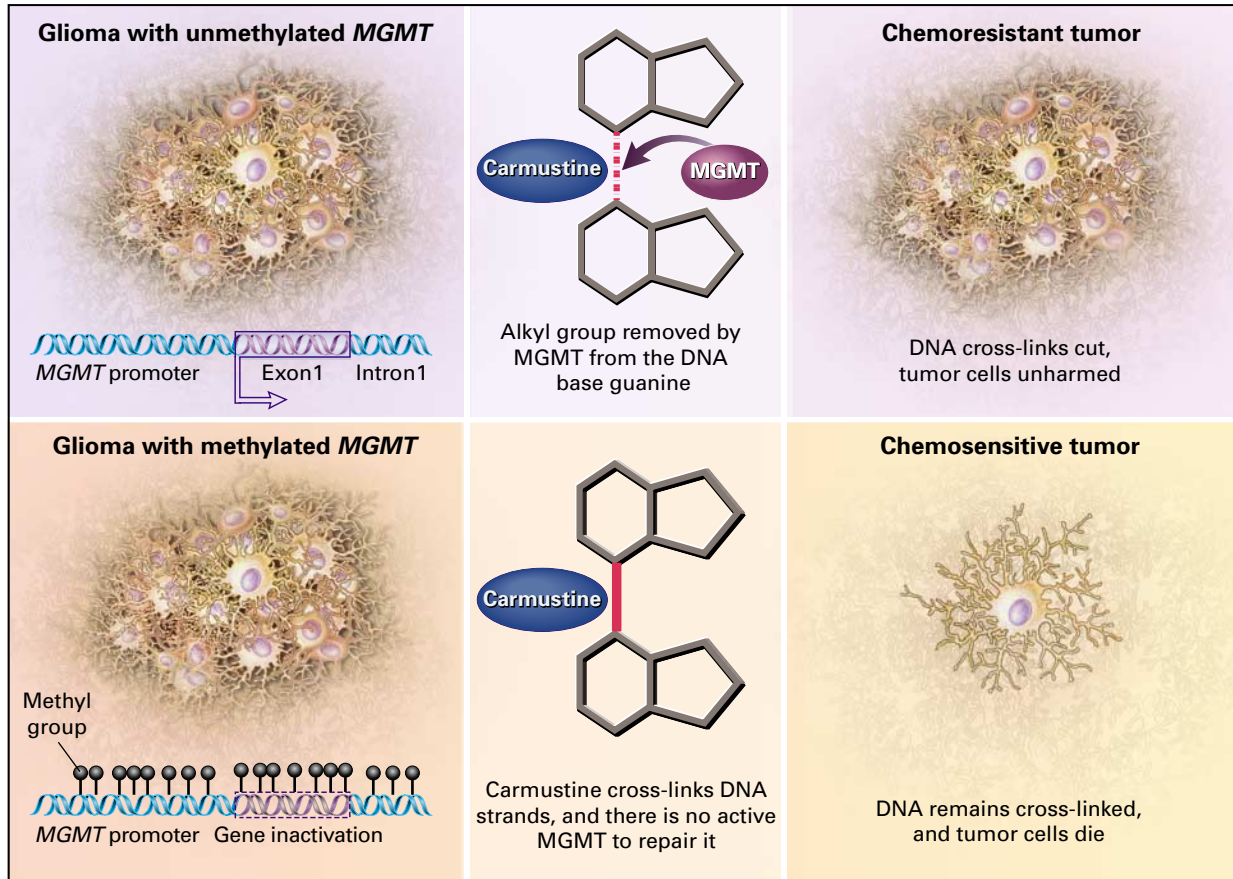


Figure 1. Mechanism of Enhanced Chemosensitivity Resulting from Epigenetic Inactivation of the DNA Repair-Gene *MGMT*. Gliomas with a methylated *MGMT* promoter and exon 1 region (circles) have transcriptional silencing of *MGMT*, leading to the loss of MGMT protein. DNA adducts produced by carmustine in these tumors are not efficiently removed, leading to tumor-cell death and drug toxicity. In contrast, gliomas with an unmethylated *MGMT* promoter and exon 1 region express MGMT protein, which removes guanine adducts from the DNA produced by the administration of carmustine (BCNU), resulting in resistance to the tumorocidal and toxic effects drug.

METHODS

Patients and Tumor Specimens

We studied specimens of brain tumors from 47 consecutive patients referred to the University Hospital of Navarre, in Pamplona, Spain, between April 1993 and November 1998. All the patients provided written informed consent. All had histologically verified tumors: 18 had an anaplastic astrocytoma, and 29 had a glioblastoma multiforme. Patients were 38 to 70 years old (median age at diagnosis, 55 years); 30 were men, and 17 were women. Tumor specimens were obtained by resection or biopsy performed before the initiation of treatment with radiation and chemotherapy and were immediately frozen and stored at -80°C . All patients were treated with intraarterial cisplatin (50 mg per square meter of body-surface area), whole-brain radiotherapy, and a median of three courses of intravenous carmustine (1,3-bis(2-chloroethyl)-1-nitrosourea, or BCNU; 100 mg per square meter) given at four-week intervals. Fifteen of the patients also underwent autologous bone marrow transplantation plus high-dose chemotherapy treatment with three doses of intravenous carmustine (300 mg per square meter) per day and one dose of intraarterial cisplatin (100 mg).

The response to treatment was evaluated after the patients had

completed therapy. A complete response was defined as the absence of any evidence of the tumor on computed tomographic (CT) and magnetic resonance imaging (MRI) scans, with no need for steroid treatment and an improvement in the patient's general condition. Patients with persistent CT abnormalities but with more than a 50 percent reduction in both the diameter and the volume of the tumor, a reduced need for steroid treatment, and a stabilized neurologic condition were considered to have a partial response. The disease was considered to have progressed if both the diameter and volume of the tumor increased by 25 percent or more of the initial measurements, if a new lesion was evident on CT or MRI scans, or if the patient's neurologic condition worsened and required an increased dose of steroids.

Analysis of Methylation

DNA was extracted according to standard protocols. Methylation patterns in the CpG island of *MGMT* were determined by chemical modification of unmethylated, but not methylated, cytosines to uracil. Methylation-specific polymerase chain reaction (PCR) was performed with primers specific for either methylated or the modified unmethylated DNA, as previously described.^{13,16} DNA (1 μg) was denatured with sodium hydroxide and modified

TABLE 1. CHARACTERISTICS OF PATIENTS WITH GLIOMAS, ACCORDING TO THE METHYLATION STATUS OF THE *MGMT* PROMOTER.

CHARACTERISTIC	UNMETHYLATED (N=28)	METHYLATED (N=19)
	no. of patients (%)	
Age		
≤50 yr	8 (29)	8 (42)
>50 yr	20 (71)	11 (58)
Sex		
Male	14 (50)	16 (84)
Female	14 (50)	3 (16)
Karnofsky score		
≤80	18 (64)	13 (68)
>80	10 (36)	6 (32)
Type of tumor		
Anaplastic astrocytoma	11 (39)	7 (37)
Glioblastoma multiforme	17 (61)	12 (63)

with sodium bisulfite. DNA samples were then purified with the Wizard DNA purification resin (Promega, Madison, Wis.), again treated with sodium hydroxide, precipitated with ethanol, and resuspended in water. Primer sequences for the unmethylated reaction were 5'TTTGTGTTTTGATGTTTGTAGGTTTTGT3' (forward primer) and 5'AACTCCACACTCTTCCAAAAACAAAACA3' (reverse primer), and for the methylated reaction they were 5'TTTCGACGTTTCGTAGGTTTTCGC3' (forward primer) and 5'GCACTCTTCCGAAAACGAAACG3' (reverse primer). The annealing temperature was 59°C. Placental DNA treated in vitro with *Sss*I methyltransferase (New England Biolabs, Beverly, Mass.) was used as a positive control for methylated alleles of *MGMT*, and DNA from normal lymphocytes was used as a negative control. Controls without DNA were used for each set of methylation-specific PCR assays. Ten microliters of each 50- μ l methylation-specific PCR product was loaded directly onto nondenaturing 6 percent polyacrylamide gels, stained with ethidium bromide, and examined under ultraviolet illumination.

Statistical Analysis

Continuous variables were compared with the use of Student's *t*-test. Contingency tables were analyzed by Fisher's exact test. Dis-

ease-free and overall survival curves were estimated by the Kaplan–Meier method and were compared with the use of the log-rank test. Multivariate survival analyses were performed with the Cox proportional-hazards model, and proportional-hazards assumptions were checked with the use of Schoenfeld residuals and graphic methods. Descriptive or stratified analyses always preceded parametric modeling in order to confirm that the assumptions underlying the models were met. The results are reported as two-sided *P* values with 95 percent confidence intervals. Analyses were performed with the use of JMP software (version 3.1, SAS Institute, Cary, N.C.) and Stata software (version 6.0, Stata, College Station, Tex.).

RESULTS

We analyzed 47 newly diagnosed grade III or IV gliomas (classified as anaplastic astrocytoma in 18 patients and as glioblastoma multiforme in 29). The characteristics of the patients are shown in Table 1. Methylation of the *MGMT* promoter was found in 19 of the 47 tumors (40 percent) (Fig. 2), a frequency similar to that found in our previous study¹³ and consistent with that in other reports.^{5,6} Methylation was not associated with the patient's age, the Karnofsky score for performance status, or the grade of the tumor ($P>0.3$ for each comparison).

In univariate analyses, methylation of the promoter was positively correlated with the clinical response and with overall and disease-free survival. Twelve of the 19 patients with methylated tumors (63 percent) had a partial or complete response to carmustine, as compared with 1 of the 28 patients with unmethylated tumors (4 percent, $P<0.001$) (Table 2). The lack of methylation was associated with a much higher risk of death (hazard ratio, 9.5; 95 percent confidence interval, 3.0 to 42.7; $P<0.001$) (Fig. 3A). In univariate analysis, no other factor had a statistically significant relation with survival. The median time to the progression of disease was 21 months for methylated gliomas and 8 months for unmethylated gliomas ($P<0.001$), and the hazard ratio associated with nonmethylation was 10.8 (95 percent confidence interval, 4.4 to 30.8) (Fig. 3B). The small number of

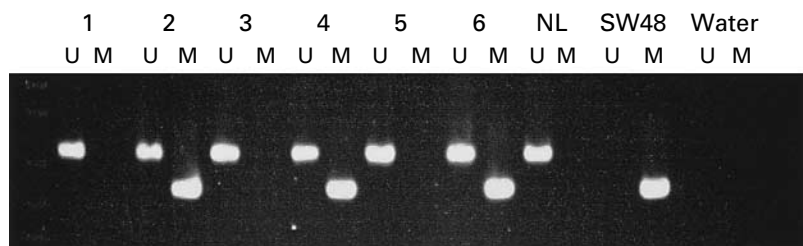


Figure 2. Methylation Status of the *MGMT* Promoter in Six Glioma Samples.

A methylation-specific PCR assay was used, with the SW48 cancer cell line as a positive control for methylation, normal lymphocytes (NL) as a negative control for methylation, and water as a negative PCR control. PBR322/*Msp* digest was used as the molecular-weight marker. U denotes the presence of unmethylated genes, and M the presence of methylated genes. Three glioma samples (2, 4, and 6) show methylation, and three samples (1, 3, and 5) are unmethylated.

TABLE 2. CLINICAL RESPONSE ACCORDING TO THE METHYLATION STATUS OF THE *MGMT* PROMOTER.*

RESPONSE	UNMETHYLATED (N=28)		METHYLATED (N=19)	
	no.	% (95% CI)	no.	% (95% CI)
Complete	0	0 (0-12)	2	11 (1-33)
Partial	1	4 (0-18)	10	53 (29-76)
No change	4	14 (4-33)	3	16 (3-38)
Progression of disease	23	82 (63-94)	4	21 (6-44)

*CI denotes confidence interval.

deaths among patients with gliomas containing a methylated promoter (four deaths) made multivariate analyses unreliable. The hazard ratio associated with a nonmethylated glioma was either unchanged or increased when other predictors were added individually to the model.

DISCUSSION

The DNA-repair enzyme *MGMT* is a key factor in resistance to alkylating agents, because the transfer of alkyl groups to *MGMT* prevents the formation of lethal cross-links in DNA.^{3,4} It has been reported that lack of *MGMT* in gliomas from patients who were treated with chloroethylnitrosoureas had only a moderate effect on overall survival, and the time to progression of disease was affected minimally or not at all.⁷⁻⁹ Using a different method to evaluate the status of the *MGMT* gene, we found a much stronger influence of the presence or absence of the enzyme. The accumulation of normal cells in the tumor, including infiltrating lymphocytes, may complicate accurate assessment of *MGMT*. The mixture of normal cells may explain, in part, the difference between the biochemical activity measured in tumor homogenates⁹ and the results of direct immunohistochemical examination of *MGMT* in tumor cells.^{7,8} The use of methylation-specific PCR permits an assessment of methylation of the *MGMT* promoter. Methylation status is an indicator of the transcriptional activity of the gene in glioma cells, and thus the presence or absence of the DNA-repair enzyme.

In our study, methylation of the *MGMT* promoter was associated with responsiveness to carmustine and an increase in overall survival and the time to progression of disease. Moreover, the methylation status of the promoter was more predictive of the outcome of carmustine treatment than the grade of the tumor, the Karnofsky performance status, or the patient's age. If further study confirms that methylation of the *MGMT* promoter predicts responsiveness to carmustine, the use of this alkylating agent might be reserved

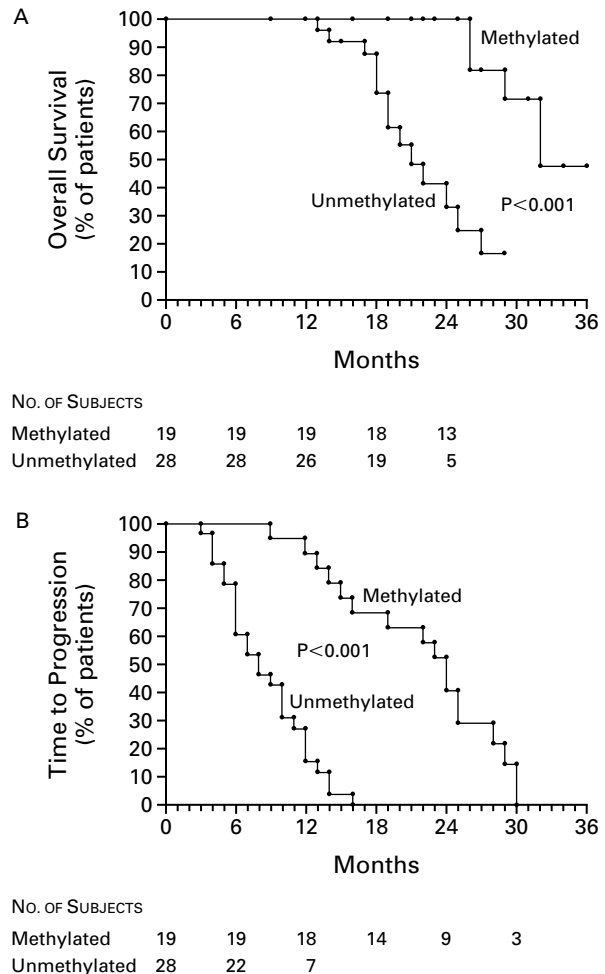


Figure 3. Overall Survival (Panel A) and Time to the Progression of Disease (Panel B) among Patients with Gliomas Treated with Carmustine, According to the Methylation Status of the *MGMT* Promoter.

Both overall survival and the time to the progression of disease were significantly greater in the group of patients with methylation of the *MGMT* promoter than in the group without methylation. The association was independent of the type of tumor, the patient's age, and the Karnofsky score for performance status.

for patients with gliomas in which the promoter is methylated. Moreover, it might be possible to increase the sensitivity of resistant tumors (those without methylation) with the use of agents that inhibit the *MGMT* enzyme. One such inhibitor, O⁶-benzylguanine,^{17,18} is being investigated for this purpose. It is a substrate for *MGMT* that inactivates the enzyme. O⁶-benzylguanine has been shown to enhance the response to alkyl nitrosoureas in vitro and in vivo.^{17,19} The use of such an agent to increase the sensitivity of gliomas to carmustine only in cases of resistant tumors might prevent the toxic effects of the combination of these drugs on normal tissues in patients who are already sensitive to carmustine.

Dr. Esteller is the recipient of an award from the Spanish Ministry of Education and Culture.

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CORRECTION

Inactivation of the DNA-Repair Gene *MGMT* and the Clinical Response of Gliomas to Alkylating Agents

Inactivation of the DNA-Repair Gene *MGMT* and the Clinical Response of Gliomas to Alkylating Agents . The following should have appeared in the article: Drs. Herman and Baylin have patented the method for the methylation-specific polymerase chain reaction described in this article. Under a license agreement between Johns Hopkins University and Virco, Ltd., Drs. Baylin and Herman are entitled to a share of the royalties received by the university on sales related to the method.

CORRECTION

The DNA-Repair Gene *MGMT* and the Clinical Response of Gliomas to Alkylating Agents

To the Editor: Esteller and colleagues (Nov. 9 issue)¹ claim that methylation of the O⁶-methylguanine-DNA methyltransferase (*MGMT*) promoter is associated with improved responsiveness to carmustine and prolonged survival in patients with high-grade gliomas. We believe there are methodologic flaws in the authors' study that call these results into question. First, the patients in the study were treated with a combination of surgery, radiation therapy, and chemotherapy with cisplatin and carmustine. We do not believe the response to carmustine is a discernible end point in this trial, because multiple treatments were administered along with carmustine.

Second, although the difference in age between the group of patients with unmethylated tumors and the group with methylated tumors was not statistically different, there was a trend toward an older age in the group with unmethylated tumors. We are not given the distribution of ages above or below 50 years. Since the prognosis worsens with each additional decade of age, such information would be useful in assessing the balance in age between the two groups.^{2,3}

Third, the median period of survival was approximately 20 months for the patients with unmethylated tumors and approximately 30 months for those with methylated tumors, as compared with an average of about 12 months in other series.^{2,3} Moreover, since no deaths occurred before 12 months, it is unlikely that the patients chosen for this study were representative of most patients with the disease. Finally, the small numbers of deaths in the two groups of patients make the results of a statistical comparison questionable.

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To the Editor: Esteller et al. describe differences in outcome based on the methylation status of the *MGMT* promoter in adults with high-grade gliomas who were treated with multimodal therapy incorporating the alkylating agent carmustine. Methylation of the *MGMT* promoter predicted an improved outcome with such therapy. This finding is potentially very important. However, neither the authors of the report nor Weinstein, in the accompanying editorial,¹ raise the question of whether methylation of the *MGMT* promoter is predictive of the outcome for patients who are not given chemotherapy as part of their primary treatment. Data from a study involving a sizable cohort suggest that the status of the *MGMT* promoter is not prognostic in patients treated with regimens that do not incorporate carmustine.² For the findings of Esteller et al. to be placed in context, not only will their study have to be replicated, but the absence of a prognostic effect in patients not given alkylating agents will also have to be confirmed.

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To the Editor: In a study of 47 patients with malignant gliomas, Esteller and colleagues report a positive correlation between inactivation of *MGMT* — by virtue of gene-promoter methylation — and survival. All the patients had undergone surgery before they received radiotherapy and chemotherapy, including carmustine. However, magnetic resonance imaging (MRI) or computed tomography was not performed immediately after surgery to determine the residual tumor volume, despite the well-known effect of the residual tumor volume on the prognosis.¹ Therefore, we are left with some uncertainty, because the results may have been biased by differences in the tumor volumes before the initiation of adjuvant chemotherapy.

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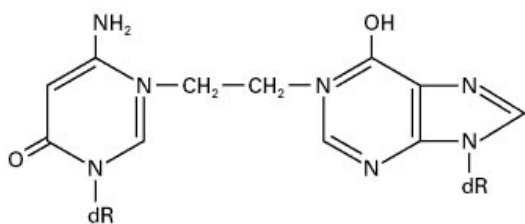
To the Editor: The methyl-excision-repair (MER)-negative phenotype, manifested by absent or decreased tumor MGMT, has been associated with a longer period of disease-free survival among patients with gliomas who are treated with carmustine. However, Esteller et al. provide definitive evidence of a correlation between survival after carmustine therapy and MGMT methylation. Their findings are thus important and provide the basis for an assay with potentially clinical value. We would, however, like to make the following comments about their report.

First, contrary to the statement by Esteller et al., the most frequent site of DNA base alkylation by monofunctional and bifunctional nitrosoureas and related alkylating agents, such as temozolomide and procarbazine, is not the O⁶-position of guanine but rather the N⁷ position of guanine and the N³ position of adenine. Second, MGMT does not repair the DNA interstrand cross-links resulting from the O⁶-chloroethylguanine adducts of nitrosoureas, as suggested in Figure 1 of the article by Esteller et al. Furthermore, in that figure, the DNA interstrand cross-link produced by carmustine is incorrectly depicted as a diguanyl cross-link. It is actually an N¹-deoxyguanosyl-N³-deoxycytidyl cross-link.¹ Its correct structure is shown in Figure 1 here.

Finally, in the absence of methylation of the MGMT gene, translational² and post-translational³ processes can alter MGMT levels and MGMT functional activity in tumors and may confound the interpretation of the methylation status of the MGMT gene. We suggest that any clinical assay based on the findings reported by Esteller et al. be validated before it is used widely in selecting patients for chemotherapy.

Figure 1. DNA Interstrand Cross-Link Formed by Carmustine, Lomustine, and Other Bifunctional Nitrosoureas.

The abbreviation dR denotes deoxyribose.



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The authors reply:

To the Editor: Dr. Quinn correctly points out that improvements in the response to treatment and survival cannot be definitively attributed to carmustine without a comparison with no treatment. The study Quinn cites showed no relation between MGMT enzyme activity and survival among patients not given chemotherapy, but it also failed to show such a relation among patients receiving chemotherapy.¹ Previous studies demonstrated a direct relation between MGMT expression in glioma cell lines and a response to the alkylating agent nimustine (ACNU), but not other chemotherapeutic agents.² Other work we have done suggests that MGMT inactivation predicts prolonged survival in patients with lymphoma who are treated with an alkylating agent (unpublished data) but not in patients with colorectal cancer who do not receive an alkylating agent (unpublished data).

Drs. Buckner and Moynihan raise several questions. Although there was a slight imbalance between the two groups in the number of patients who were over 50 years old, the age distribution did not differ statistically. In a univariate analysis, age was minimally associated with progression-free survival (hazard ratio for the risk of progression, 0.99) and overall survival (hazard ratio for the risk of death, 0.92); the associations were not statistically significant. Most important, the association of MGMT methylation with overall and progression-free survival was independent of age, as indicated in the legend to Figure 3 of our article. Differences in survival between our study and others may be due to differences in treatment regimens, performance status, and tumor grade (with a higher prevalence of grade 3 tumors in our study); however, these differences do not change the conclusions of our study. Our statistical analysis took into account the size of the sample.

We regret that in our article we did not clearly state that we obtained MRI scans for all patients after surgery in order to provide a base line for evaluating the response to treatment, and we thank Dr. Schlegel for allowing us to make this clarification. We thank Ali-Osman et al. for clarifying issues related to the chemistry of alkylating agents. Although other sites of DNA base alkylation may be more frequent, the O⁶ position appears to be most important for sensitivity to alkylating

agents and the adduct most closely related to *MGMT* expression.³ Although *MGMT* does not repair cross-links, it prevents their formation by the removal of alkyl groups. Finally, translational and post-translational changes in *MGMT* that were not determined by examination of promoter-region methylation would be relevant only in the tumors with unmethylated *MGMT* promoters, which transcribe the gene. Such changes, if they had been present, would not have led to the observed association.

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The editorialist replies:

To the Editor: Dr. Quinn is correct when he points out that the value of the findings reported by Esteller et al. would be enhanced by comparison with data from a group of patients with gliomas who were not treated with carmustine (or other alkylating agents). If methylation of the *MGMT* promoter region in tumor samples from such patients were not correlated with improved overall and disease-free survival, that finding would strongly support the hypothesis of a causal relation between the activity of carmustine and the methylation. Going one step further, a survey of the methylation status of other promoter regions in the glioma samples analyzed by Esteller et al. would indicate whether the putative relation with methylation was specific to the *MGMT* promoter. Nonetheless, the authors' principal conclusion that "methylation of the *MGMT* promoter in gliomas is a useful predictor of the responsiveness of the tumors to alkylating agents" stands without such additional studies. One could argue that the term "alkylating agents" is too broad, since the data are only for the nitrosourea carmustine, but the data do identify a "useful predictor" if one assumes (on the basis of the clinical course and timing) that the observed responses were actually due to the treatment with carmustine. Dr.

Quinn's critique illustrates the difficulty of establishing pharmacogenomic causality, especially in the clinical setting.

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